The Effect of Protein Conformation Change from α_{II} to α_{I} on the Bacteriorhodopsin Photocycle

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ABSTRACT The bacteriorhodopsin (bR) photocycle was followed by use of time-resolved Fourier-transform infrared (FTIR) spectroscopy as a function of temperature (15–85°C) as the $\alpha_{II} \rightarrow \alpha_{I}$ conformational transition occurs. The photocycle rate increases with increasing temperature, but its efficiency is found to be drastically reduced as the transition takes place. A large shift is observed in the all-*trans* $\leftrightarrow$ 13-*cis* equilibrium due to the increased stability of the 13-*cis* isomer in α_{I} form. This, together with the increase in the rate of dark adaptation as the temperature increases, leads to a large increase in the 13-*cis* isomer concentration in bR in the α_{I} form. The fact that 13-*cis* retinal has a much-reduced absorption cross-section and its inability to pump protons leads to an observed large reduction in the concentration of the observed photocycle intermediates, as well as the proton gradient at a given light intensity. These results suggest that nature might have selected the α_{II} rather than the α_{I} form as the helical conformation in bR to stabilize the all-*trans* retinal isomer that is a better light absorber and is capable of pumping protons.

INTRODUCTION

Bacteriorhodopsin (bR), the protein that spans the cell membrane of *Halobacterium salinarium* (Stoeckenius and Lozier, 1974), functions as a light-driven proton pump. The purple membrane (PM) consists of a two-dimensional crystal of bR trimer structure that further organizes the individual bR molecules into a hexagonal lattice (Henderson et al., 1990). It has only one polypeptide chain of 248 amino acid residues with known sequence, and a retinal chromophore that binds to the ϵ-amino group Lys-216 through a protonated Schiff base. Upon absorbing a photon, bR undergoes a photocycle that consists of a series of photointermediates (Stockburger et al., 1979; Birge, 1981; Mathies et al., 1991) with lifetimes ranging from as short as a half-picosecond to tens of milliseconds. As a result, a proton is pumped across the membrane from the cytoplasmic to the extracellular side creating such an electrochemical proton gradient, which is used in the metabolic transformation of ADP into ATP.

Bacteriorhodopsin is one of the most rugged membrane proteins known. It melts at a temperature over 50°C higher than that of most mammalian proteins. Its secondary structure has been well established, with seven α-helices across the membrane. It was proposed (Krimm and Dwivedi, 1983) that the bR transmembrane helices are α_{II}-helical in nature, different from the normal α_{I}-helical structure found in most polypeptides and proteins. Strong evidence has been found to support this proposal, such as infrared (Taneva et al., 1995; Torres et al., 1995; Wang and El-Sayed, 1999), Raman (Vogel and Gaertner, 1987), ultraviolet-circular dichroism (UV-CD) (Gibson and Cassim, 1989), and ^{13}C nuclear magnetic resonance (NMR) studies (Tuzi et al., 1994). The α_{II}-helical structure has its dihedral angles (ϕ and ψ) different from those in α_{I}-helical conformation, leading to an outward projection of the C═O groups from the helix axis accompanied by an inward tilting of the N—H groups. In α_{II}-helical structure, the H-bond length is slightly longer than that in α_{I}. Vibrational normal mode analysis for polyalanine (Krimm and Dwivedi, 1983) predicts significant frequency differences between the standard α_{I}- and the modified α_{II}-helical conformation. The amide I mode (essentially C═O stretching) in the α_{II}-helical structure is at 1658 cm^{-1}, which is ~10 cm^{-1} blue-shifted from that of α_{I}.

Bacteriorhodopsin has two thermal transitions (Jackson and Sturtevant, 1978; Hiraki et al., 1981; Wang and El-Sayed, 1999), one the reversible premelting transition, with a melting temperature (T_m) at ~78°C, and the other one is the main, irreversible melting transition, with a T_m of ~96°C. It is also found that the main melting temperatures are pH and metal cation content-dependent (Kresheck et al., 1990). The temporal behavior and dynamics of the reversible premelting transition have recently been determined (Wang and El-Sayed, 1999), by using time-resolved FTIR spectroscopy and laser-induced temperature-jump techniques. Two main processes have been identified, in which one process involves a fast α_{II} to α_{I} conversion that occurs in the tens of nanosecond time domain, and the other process is a large conformational change that allows water to penetrate the hydrophobic domains of the protein, which occurs on ~400-ns time scale.

The protein conformational change during the photocycle of bR can be revealed in the amide I frequency region. At room temperature, large global conformational changes occur upon M and N formation, as revealed by diffraction studies (Dencher et al., 1989; Koch et al., 1991; Nakasako et al., 1991; Subramaniam et al., 1993). Two prominent peaks appearing at 1669(−)/and 1649(+) cm^{-1} have been

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observed in the N-bR FTIR difference spectra (Pfefferle et al., 1991; Braiman et al., 1991; Ormos et al., 1992; Hessling et al., 1993; Kandori, 1998), in the case of bR in H_2O.

Because bR ground state conformation varies from α_{II} to α_I as temperature increases (Taneva et al., 1995; Torres et al., 1995; Wang and El-Sayed, 1999), and most helical proteins have the α_I conformation, questions then arise are whether the α_I conformation would allow bR to carry out its proton pumping function and what the conformational change in the photointermediate is during its photocycle.

In the present study the photocycle of bR is initiated with a nanosecond laser excitation at 532 nm in the temperature range 15–85°C, within which the $\alpha_{II} \rightarrow \alpha_I$-helical conformation change occurs. Step-scan time-resolved FTIR technique is used to follow the photocycle and the protein conformational changes in the spectral regions of retinal C═C stretching vibrational modes and protein amide modes. It is found that as the structure of the protein changes from α_{II}- to α_I-helices, there is an increase in the equilibrium concentration of the 13-*cis* retinal at the expense of the all-*trans* isomer. This, together with the known increased rate of bR dark adaptation at higher temperature, the much lower absorption cross-section of the 13-*cis* isomer, and its inability to pump protons all lead to a large reduction in the photocycle probability.

EXPERIMENTAL

Sample

PM was isolated from the *Halobacterium salinarium* strain ET1001 according to a general procedure (Stoeckenius and Lozier, 1974). PM fragments containing bR molecules are suspended in D_2O by washing the membrane 6–8 times with D_2O to reach a thorough D_2O/H_2O (D/H) exchange. The initial pH of the concentrated suspension before D/H exchange was typically 6.8. A 50-μm path-length IR cell with two CaF_2 plates and a Teflon spacer was used for bR. Samples have an optical density at 568 nm of ~0.8. Sample temperature (from 20°C to 85°C) is controlled by a thermostated bath system (RET-100, NESLAB Instruments, Inc., Union City, CA). UV-Vis spectra are also taken at each temperature by using the same IR cell and temperature controller.

Time-resolved FTIR spectroscopy

The step-scan FTIR measurements were carried out on a Bruker IFS 66/S spectrometer as described previously (Wang and El-Sayed, 1999). Data were taken with a temporal resolution of 1–10 μs and a spectral resolution of 4 cm^{-1}; 10–15 co-additions were used to improve the signal-to-noise ratio. Samples were photoexcited at 532 nm by an Nd:YAG laser (Quantum-Ray DCR-3, 10 Hz repetition rate), with laser energy of 3 mJ/pulse.

RESULTS AND DISCUSSION

Fig. 1 shows the visible absorption spectra of light-adapted bR in D_2O. These spectra show a blue-shift from its maximum absorption at 568 nm at 30°C to ~537 nm at 85°C, and a large decrease in the band intensity. Both the original spectra (Fig. 1 *A*) and the difference spectra (Fig. 1 *B*, spectra at a certain temperature minus that at 30°C), show a sudden and larger decrease in the intensity between 60 and 70°C, the onset of the reversible α_{II} to α_I transition. The integrated absorption as a function of temperature is given in Fig. 2. An abrupt change in the integrated absorption is observed between 60 and 70°C. The observed blue-shift in the visible absorption spectrum of bR at high temperature indicates the formation of the 13-*cis* retinal isomer.

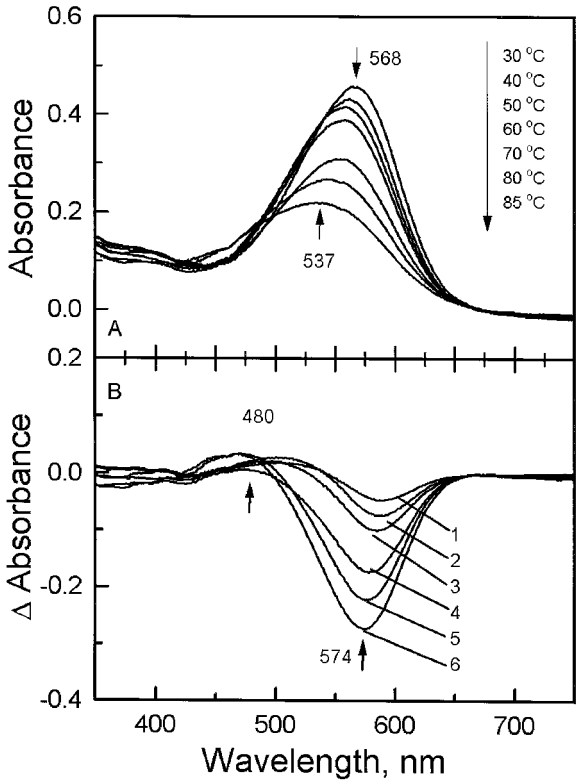

FIGURE 1 Visible absorption spectra of bR in D_2O as a function of temperature. (*A*) Original spectra and (*B*) difference spectra. The temperature ranges from 30 to 85°C as indicated. As temperature increases a blue-shift is observed, and the maximum absorption changes from 568 nm at 30°C to 537 nm at 85°C, which is also accompanied by a decrease in the absorption integrated intensity (*A*). A controlled experiment shows that this blue-shift is reversible, i.e., after cooling the sample at 85°C back down to room temperature, both the intensity and the wavelength of the maximum recover their values before heating. This is again inconsistent with the reversibility of the α_{II} to α_I transition.

Fig. 3 shows the time-resolved FTIR difference spectra in the frequency region between 1800 and 1450 cm^{-1}, at temperatures ranging from 15 to 85°C. In the difference spectra the positive bands originate from the photointermediates, whereas the negative bands result from the ground state depletion as bR molecules absorb light and go through the photocycle. As the temperature increases, the rate of the formation and decay of the different intermediate increase, as does the rate of dark adaptation. This leads to a decrease in the intensity of the difference spectrum observed with increasing temperature. Here the spectral feature tells us that

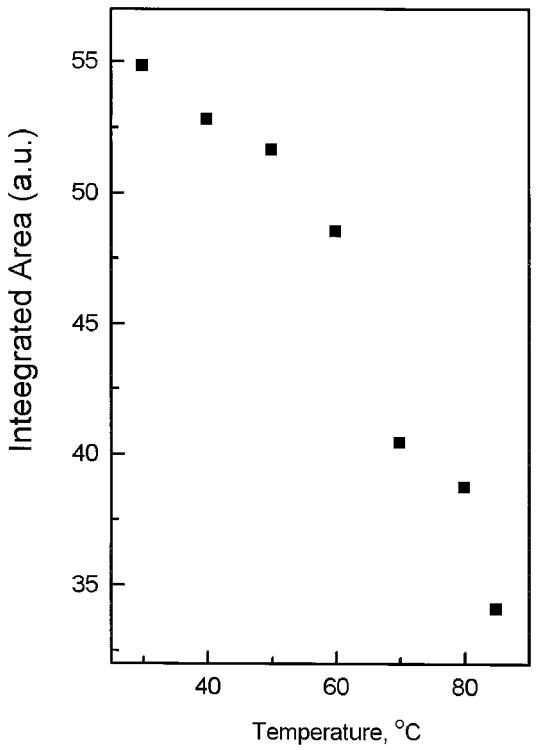

FIGURE 2 The integrated absorption of bR in D_2O as a function of temperature. A sudden change is observed between 60 and 70°C, the onset of the premelting α_{II} to α_I transition.

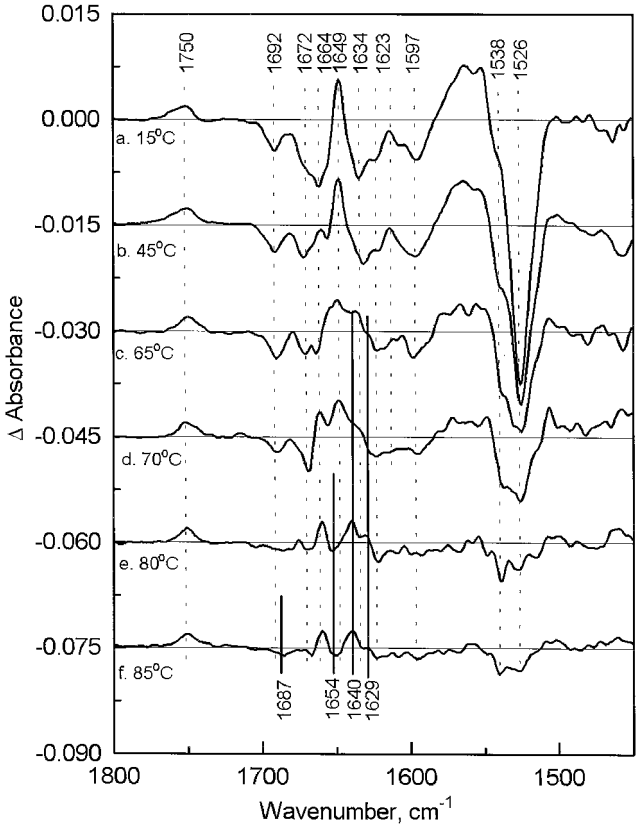

FIGURE 3 Time-resolved FTIR difference spectra of M/bR in D_2O at various temperatures and integration time range after laser excitation, as indicated. The spectral ranges from 1800 to 1450 cm^{-1}, at the resolution of 4 cm^{-1}. (*a*) 15°C, 500-1000 μs; (*b*) 45°C, 80–580 μs; (*c*) 65°C, 30–350 μs; (*d*) 70°C, 10–350 μs; (*e*) 80°C, 5–350 μs; and (*f*) 85°C, 1–350 μs. All spectra are normalized at the C═O stretch vibration mode at 1750 cm^{-1}.

we have a mixture of mainly M and small amount of N. The spectrum at 15°C is in good agreement with that previously reported (Weidlich and Siebert, 1993), especially in the region of amide I, which is quite different from the spectra obtained in H_2O.

Different average time domains are chosen at different temperature to give (M + N)-bR spectra. The positive band at 1750 cm^{-1} of bR in D_2O (C═O stretching mode of Asp-85 in D_2O, which is at 1763 cm^{-1} in H_2O), indicates the formation of the M intermediate as the deuteron is transferred from the deuteronated Schiff base to $(Asp\text{-}85)^-$. Therefore, the spectra are normalized with respect to the band at 1750 cm^{-1} in Fig. 3. It is found that as the temperature increases, a shorter rise time of the 1750 cm^{-1} band is observed. Therefore, the M intermediate reaches its maximum intensity within 500-1000 μs at 15°C (Fig. 3 *a*), whereas it is within 1–350 μs at 85°C (Fig. 3 *f*). Measurement of the bR photocycle above 85°C seems impossible, as bR is found to be bleached very quickly upon excitation with the 532-nm laser.

It is also found that the band shape and frequency for the C═C stretching mode of the all-*trans* retinal change as the temperature changes. Thus the main frequency observed at ~1526 cm^{-1} at temperatures below 65°C (Fig. 3, *curves a–c*), becomes a doublet at 1526 and 1538 cm^{-1} above 65°C (Fig. 3, *curves c–f*). This is accompanied by a large decrease in the band intensity of these bands even if normalized with respect to the band at 1750 cm^{-1}. The doublet of the C═C stretching mode at 1538 and 1526 cm^{-1} can be assigned to the ground-state bleach of the 13-*cis* and all-*trans* retinal, respectively.

In our (M + N)-bR difference spectra at various temperatures (as shown in Fig. 1), the following changes in the amide I band region are observed, in the form of negative and positive bands: 1692(−), 1672(−), 1664(−), 1634(−), 1623(−), and 1649 cm^{-1}(+) at 15°C (Fig. 1 *A*). This is in good agreement with a previous report (Weidlich and Siebert, 1993). At 85°C, there are bands at 1687(−), 1667(−), 1654(−), 1623(−), 1664(+), 1640(+), and 1629(+) cm^{-1} (Fig. 3 *f*). In the amide I region, new bands that most likely are due to α_I begin to appear at low frequency, even though the temperature dependence of individual bands may cause them to increase in frequency. This has been summarized in Table 1. It is also noted that at all temperatures a negative band at 1623 cm^{-1} is observed, which is due to the C═ND^+ stretching vibration in the ground state. A positive band at 1613 cm^{-1} results from the

TABLE 1 Transient spectral change in the amide I region at various temperature

Temp (°C)	Bleach (cm^{-1})						Absorption (cm^{-1})				
15	1692,	1672,	1644,	—	1634,	1623	—	1649,	—	—	1613
45	1692,	1672,	—	—	1634,	1623	1664,	1649,	—	—	1613
65	1689,	1672,	—	—	—	1623	1664,	1649,	—	1634,	1613
70	1689,	1667,	—	1654,	—	1623	1664,	1649,	1640,	1629,	1613
80	1687,	1667,	—	1654,	—	1623	1664,	—	1640,	1629,	1613
85	1687,	1667,	—	1654,	—	1623	1664,	—	1640,	1629,	1613

The bleached bands are for bR ground state where the positive bands are for the photointermediate.

C═N stretching vibration in the intermediate spectrum. At higher temperature the band becomes less significant (Fig. 3, *d–f*) due to the large reduction in all the spectral changes. As temperature increases, it has been reported (Taneva et al., 1995; Torres et al., 1995; Wang and El-Sayed, 1999) that bR undergoes a transition from α_{II} to α_I, with the amide I band shifted from 1667 cm^{-1} for α_{II} to 1652 cm^{-1} for α_I. Consequently, complicated spectral features in this frequency region are expected for the (M + N)-bR difference spectrum, as shown in Fig. 3, *b–f*. Possible assignment of these bands at 85°C is tentatively made in the following way: 1667(−)/1664(+) cm^{-1} for the α_{II}-helical conformation; 1654(−)/1640(+) cm^{-1} for the possible α_I-helices. However, considering that the origin of the C═O in the amide I region is due to the protein polypeptide backbone (Kandori, 1998), as protein conformation changes from α_{II} to α_I-helices, the exposure of the hydrophobic domain to the aqueous medium might also cause the shift of those C═O stretching vibrations.

Fig. 4 gives the ratio (R) of the band intensity at 1538 and 1526 cm^{-1} with respect to that of the C═O band at 1750 cm^{-1} as a function of temperature. It is noted that the value of R remains almost constant in the temperature region from 25 to 60°C. The ratio R starts to increase when the temperature is above 65°C. Although the error bars in Fig. 4 give uncertainty in the R-values, it is still quite obvious that the change in R occurs in the transition temperature range of 70 to 80°C, as reported before (Wang and El-Sayed, 1999). This is an indicator of the transformation of chromophore from all-*trans* retinal to 13-*cis* retinal.

A Fourier-transform Raman study (Jas and Johnson, 1994) of light-adapted bR at at ~78°C shows that the C═C stretching mode is blue-shifted from 1522 cm^{-1} (the all-*trans* form) to 1535 cm^{-1}. Their finding is in agreement with our observation here (Fig. 3), the doublet of the C═C stretching mode at 1538 and 1526 cm^{-1} appearing at high temperatures due to the ground-state bleaching of the 13-*cis* and all-*trans* retinal.

Based on our results here, it seems that there is a correlation between the retinal configuration and protein conformation, i.e., bR in the form of α_{II}-helical prefers all-*trans* retinal, whereas 13-*cis* retinal is preferred in the α_I-helices. It was previously reported (Schulte et al., 1995) that in the Raman spectrum of dark-adapted bR the deconvolution of the ethylenic mode into bands assigned to the all-*trans* (1526 cm^{-1}) and 13-*cis* (1534 cm^{-1}) isomers yields a 13-*cis* to all-*trans* ratio equal to 1 at ambient pressure. Near-IR (NIR) Raman spectroscopy is used as an in situ probe of the chromophore conformation to study the light-to-dark adaptation process in bR at variable pressure (Schulte and Bradley, 1995). Detailed spectroscopic evidence is presented to show that at high pressure the equilibrium is shifted toward the 13-*cis* isomer and that the light-to-dark adaptation kinetics are accelerated. In another study (Tsuda and Ebrey, 1980), the equilibrium constant K ($K = [bR_{all\text{-}trans}]/[bR_{13\text{-}cis}]$), determined by the retinal iso-

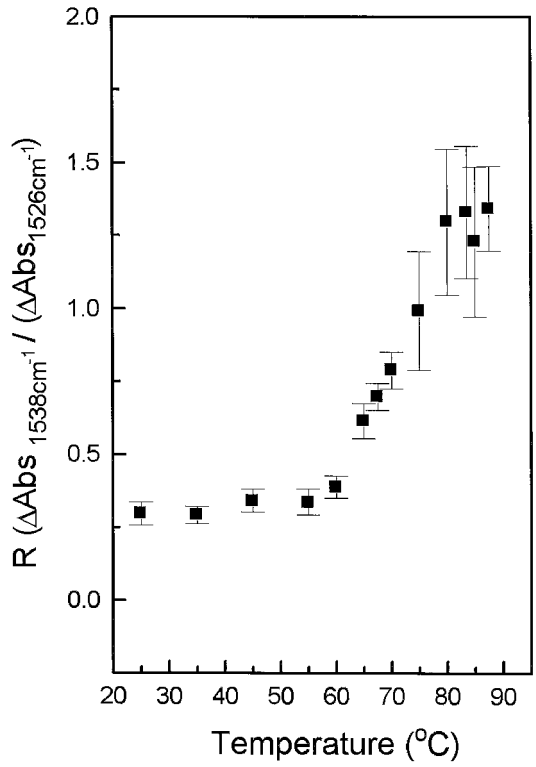

FIGURE 4 The ratio of the bleached absorbance change at 1536 cm^{-1} (13-*cis* retinal) and 1527 cm^{-1} (all-*trans* retinal) in bR ground state at various temperatures. It is noted that the value of R keeps almost constant in the temperature region from 20 to 60°C, and starts to increase as temperature is higher than 60°C. This transition corresponds to the reported (Wang and El-Sayed, 1999) reversible premelting transition from α_{II} to α_I.

meric composition, is found to be smaller at higher pressure ($K = 0.25$ at 3 kbar). Very importantly, a recent study has shown that there is an observed high-pressure induced conformational change from of α_{II} to α_I under pressure as high as a few kilobars (Barnett et al., 1997).

The above observations and possible conclusions can be summarized as follows:

1. The visible absorption shows that as the temperature increases, the total absorption integrated intensity decreases with a blue shift toward the 13-*cis* absorption maximum. There is a more rapid decrease in the absorption above 60°C, which is the onset of the $\alpha_{II} \rightarrow \alpha_I$ transition. This suggests that as temperature increases, the all-*trans* $\leftrightarrow$ 13-*cis* equilibrium shifts in favor of the 13-*cis*. Such a shift in the equilibrium mixture is enhanced when the protein changes its conformation from the $\alpha_{II} \rightarrow \alpha_I$ form.
2. This conclusion is supported by the time-resolved FTIR spectra. As temperature increases, the C═C stretching vibration of the 13-*cis* at 1538 cm^{-1} increases in intensity relative to that of the all-*trans* C═C vibration at 1526 cm^{-1}. A sudden increase in the ratio starts at ~60°C and continues through the known pretransition temperature range (Wang and El-Sayed, 1999).
3. The overall transient FTIR spectra of the bR photocycle decreases with temperature, which cannot be quantified, due to the fact that the photocycle rate increases with temperature, therefore a decrease of the bleach and photointermediate absorption can be observed above 65°C. This suggests that the proton pump photocycle is diminished if the bR protein has the α_I form than in the α_{II} form. From conclusions 1 and 2 above, this can be attributed to the fact that all-*trans* retinal is unstable compared to the 13-*cis* in the α_I form. Since the 13-*cis* retinal does not go through the proton pump cycle, an observed decrease in the concentration of the photocycle intermediates results. An additional cause of the decrease in the photocycle intermediates is the known increase in the rate of dark adaptation with temperature. It might also increase in the α_I conformation of the protein. This leads to the concentration increase of the 13-*cis* retinal in the mixture. Furthermore, it is possible that the photostationary mixture of the all-*trans* excited state and K shifts toward the all-*trans* form. This decreases the efficiency of the photoisomerization process as well. However, we have no results to suggest or eliminate this possibility from these long-time experiments. A femtosecond experiment is now in process to examine this possibility.
4. As bR changes its secondary structure from the α_{II} to α_I in the 60–80°C range, a complicated spectral feature is observed in the amide I region, indicating the variation of protein ground-state conformation as a result of the temperature change, and the protein conformational change in its intermediates as a result of the photocycle.

In conclusion, under the same light intensity, time-resolved FTIR spectroscopy shows that the concentration of the different intermediates of the bR photocycle greatly diminishes as the protein changes its conformation from the α_{II} to α_I in the 60–80°C temperature range. This is found to result from the shift of the all-*trans* to 13-*cis* equilibrium. This is confirmed from the observed enhanced reduction of the optical absorption of the all-*trans* retinal and the appearance of the much weaker absorption of the 13-*cis* form. This is also supported by an increase in the intensity of the bleach band of the 13-*cis* at 1538 cm^{-1}, as α_{II} is transformed into α_I. The above finding could explain why nature has evolved the α_{II} conformation for bR rather than the common α_I conformation found in most other helical proteins. The α_{II} conformation stabilizes the all-*trans* form of the retinal. This allows for the maximum absorption of light and the ability to pump protons in its photocycle, as the 13-*cis* form does not pump protons.

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